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In Vivo Interactions of RNA Polymerase II with Genes of *Drosophila melanogaster*

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We describe a method for examining the in vivo distribution of a protein on specific eucaryotic DNA sequences. In this method, proteins are cross-linked to DNA in intact cells, and the protein-DNA adducts are isolated by immunoprecipitation with antiserum against the protein. Characterization of the DNA cross-linked to the precipitated protein identifies the sequences with which the protein is associated in vivo. Here, we applied these methods to detect RNA polymerase II-DNA interactions in heat-shocked and untreated *Drosophila melanogaster* Schneider line 2 cells. The level of RNA polymerase II associated with several heat shock genes increased dramatically in response to heat shock, whereas the level associated with the copia genes decreased, indicating that both induction of heat shock gene expression and repression of the copia gene expression by heat shock occur at the transcriptional level. Low levels of RNA polymerase II were present on DNA outside of the transcription units, and for at least two genes, hsp83 and hsp26, RNA polymerase II initiated binding near the transcription start site. Moreover, for hsp70, the density of RNA polymerase II on sequences downstream of the polyadenylate addition site was much lower than that observed on the gene internal sequences. Examination of the amount of specific restriction fragments cross-linked to RNA polymerase II provides a means of detecting RNA polymerase II on individual members of multigene families. This analysis shows that RNA polymerase II is associated with only one of the two cytoplasmic actin genes.

Specific protein-DNA interactions have a critical role in gene expression. In eucaryotes, these interactions are likely to be strongly influenced by the structure of chromatin, the complex nucleoprotein component of the nucleus in which the DNA is packaged (25, 32). Both the puffing at transcriptionally active loci of polytene chromosomes (4) and the increased nuclease sensitivity of transcribed DNA in isolated nuclei (45) indicate that the structure of transcribed chromatin differs from that of nontranscribed regions. Immunofluorescence analyses (37) and chromatin fractionation techniques (31, 46) indicate that the protein compositions of transcribed and nontranscribed chromatin are different. The DNase I-hypersensitive sites detected at promoters of many genes even before gene expression (6) indicate that these regions of the chromosome may be primed for interacting with specific proteins. Thus, full understanding of the role of specific protein-DNA interactions in the cell requires consideration of their interactions with DNA that is packaged as chromatin rather than naked DNA.

We have recently developed a method for examining the in vivo distribution of RNA polymerase on bacterial DNA (13). Here, we use modifications of this method to map the in vivo distribution and relative density of RNA polymerase II on several *Drosophila melanogaster* genes in heat-shocked and non-heat-shocked cell cultures. Since a brief heat shock treatment of cells induces expression of a few genes and represses expression of most genes (1), we can measure changes in RNA polymerase II-DNA interactions that accompany changes in transcription. The good correlation between our measurements and transcript measurements indicates that the method can be used to assess the existence of specific protein-DNA interactions in vivo.

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MATERIALS AND METHODS

Plasmid DNA and other reagents. The plasmids containing the following *D. melanogaster* DNA segments are described elsewhere: DmrY22 containing ribosomal DNA (5); 132E3 and 56H8 containing hsp70 (27); DmA2 containing actin (11); aDm4.46 containing hsp83 (28); 202.7 containing hsp23, hsp26, and hsp27 (3); cDm500 containing histone (22); cDm1142 containing copia (9).

Polyclonal antisera to *D. melanogaster* RNA polymerase II were generously provided by A. Greenleaf. Antiserum to RNA polymerase II is described in reference 15, and antiserum to the 215,000-dalton subunit is described in reference 44.

Cross-linking of RNA polymerase II to DNA in *D. melanogaster* cell culture. Schneider line 2 cells (35) were grown in spinner flasks to densities of between 7×10^6 and 1×10^7 cells per ml at 22°C in Shields and Sang medium (36) supplemented with 12% fetal calf serum and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 6.8]). Except where stated otherwise, cultures were heat shocked by incubation at 37°C for 30 min. Heat-shocked and non-heat-shocked cells in medium were chilled 5 min on ice and then irradiated for 10 min at 0°C in a vessel in which the sample did not exceed a depth of 0.5 cm. The source of UV light was an inverted transilluminator (Chromato-Vue model C-61; UV Products) that was placed 10 cm above the cells with its filter removed, providing a light intensity of 4×10^4 erg/cm^2 per s.

Preparation of protein-DNA adducts. Nuclei were prepared from cells in the following way. Cells were collected by centrifugation for 10 min in a clinical centrifuge and suspended at no more than 10^8 cells per ml in 100 mM KCl–50

mM NaCl–5 mM $MgCl_2$–10 mM Tris-hydrochloride (pH 7.4)–0.5 mM phenylmethylsulfonyl fluoride at 0°C. A 1/10 volume of fresh 10% Nonidet P-40 (NP40) was added, and the mixture was vortexed intermittently for 5 min. The nuclei were collected by centrifugation for 10 min at 4°C in a clinical centrifuge.

When fewer than 3×10^8 nuclei were being processed, the nuclei were suspended in 900 μl of 100 mM NaCl–10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride–0.1% NP40, 100 μl of 20% Sarkosyl was added, and the viscous lysate was sheared by 10 passages through a 26-gauge needle. The sample was layered onto a CsCl block gradient consisting of 1.5 ml of CsCl at 1.75 g/cm^2, 1 ml of CsCl at 1.5 g/cm^2, and 0.9 ml of CsCl at 1.3 g/cm^2. Each layer of CsCl also contained 0.5% Sarkosyl and 1 mM EDTA (pH 7.5). The samples were centrifuged for 20 h at 30,000 rpm in an SW60 rotor at 20°C. Fractions (15 drops) were collected from the bottom of the tube through a 20-gauge needle. Samples with refractive indexes (at 22°C) between 1.4000 and 1.3800 were pooled, dialyzed into 0.5% Sarkosyl–50 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA, and then stored at −70°C.

When more than 3×10^8 nuclei were being processed, the nuclei were suspended in 9 ml of 150 mM NaCl–10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride–0.1% NP40, 1 ml of 20% Sarkosyl was added, and the viscous lysate was sheared for 60 s with a polytron (type pt 10 20 350D, Nr 4506; Brinkmann Instruments Inc.) set at the maximum level at which the sample did not foam. The sheared lysate was centrifuged for 15 min in a clinical centrifuge at 4°C to remove any insoluble material. 10 ml of supernatant was layered onto a CsCl block gradient consisting of 18.5 ml of CsCl at 1.75 g/cm^2, 6 ml of CsCl at 1.5 g/cm^2, and 3.5 ml of CsCl at 1.35 g/cm^2. Each layer also contained 0.5% Sarkosyl and 1 mM EDTA (pH 7.5). The samples were centrifuged for 36 h at 26,000 rpm in an SW28 rotor at 20°C. Fractions (1 ml) were collected with a 20-gauge needle that was inserted through the bottom of the tube slightly off center so as to avoid the pellet on the bottom of the tube. Fractions with refractive indexes of 1.385 to 1.400 contained DNA on the basis of an ethidium bromide drop assay (34). These fractions were pooled, dialyzed into 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA–0.5% Sarkosyl, and stored frozen at −70°C.

Restriction enzyme digestions in the presence of detergents. Samples were diluted into restriction enzyme buffer as specified by the supplier except for the additional presence of 0.2% Sarkosyl and 0.8% NP40. Each sample was incubated for 10 min at 37°C before the addition of restriction enzymes. For a sample of DNA derived from 10^7 cells (~10 μg of DNA), enough restriction enzyme was added so that the units of enzyme multiplied by the time of digestion at 37°C was equal to at least 50. In general, digestion times did not exceed 24 h. Reactions were stopped by adding 0.2 M EDTA to a final concentration of 20 mM, and samples were then either immunoprecipitated immediately or frozen at −70°C.

Not all restriction enzymes were active in the presence of the detergents. Those that we found to be inactive were *Pst*I, *Hha*I, and *Xba*I. Those that we found to be at least partially active were *Eco*RI, *Bam*HI, *Pvu*II, *Xho*I, *Stu*I, *Sal*I, *Hin*dIII, *Hin*fI, and *Hae*III.

Immunoprecipitations of protein-DNA adducts per 10^7 cells. Sonicated samples from 10^7 cells were pretreated with 2.5 μl of protein A-Sepharose as described previously (13) for 1 h to remove DNA that nonspecifically associates with the protein A-Sepharose pellet. The protein A-Sepharose was maintained in suspension by shaking the samples on an Eppendorf shaker and then collected by centrifugation for 60 s in an Eppendorf microfuge. The supernatant was treated with 0.5 μl of anti-RNA polymerase II antiserum for 3 to 12 h at 0°C to bind RNA polymerase II-DNA adducts and then with 2.5 μl of protein A-Sepharose for 1 to 3 h at 0°C to bind immune complexes. Bound immune complexes were collected by centrifugation for 60 s in the microfuge and then washed six to eight times at 0°C as described previously (13) with wash buffer (0.1 M Tris-hydrochloride [pH 9.0], 0.5 M LiCl, 1% NP40, 1% deoxycholate). Immune complexes were eluted from the protein A-Sepharose with four 10-min rinses with 100-μl (room temperature) portions of elution buffer (1% sodium dodecyl sulfate, 50 mM $NaHCO_3$) which contained either 1 μg of sonicated salmon sperm DNA per ml for samples to be dot blotted or 0.1 μg of φX174 DNA per ml for samples to be nick translated.

Immune complexes containing restriction-cut DNA were collected with Formalin-fixed staphylococcus A cells (Pansorbin; Calbiochem-Behring) instead of protein A-Sepharose, since protein A Sepharose did not efficiently bind the immune complexes containing these long DNA molecules. Before being used, the Formalin-fixed staphylococcus A cells were collected by centrifugation (either 45 s in an Eppendorf microfuge or at 6,500 × *g* for 15 min) and suspended at 20% (vol/vol) in 0.2% Sarkosyl–1 mM EDTA–10 mM Tris-hydrochloride (pH 7.4). The samples of protein-DNA adducts from 10^7 cells were pretreated with 10 to 25 μl of Formalin-fixed staphylococcus A cells for 1 h and then cleared of the Formalin-fixed staphylococcus A cells by centrifugation for 45 s in the microfuge. The supernatant was treated with 0.5 μl of anti-RNA polymerase II antiserum for 3 to 12 h at 0°C to bind RNA polymerase II-DNA adducts and then with 2.5 μl of Formalin-fixed staphylococcus A cells for 1 to 3 h at 0°C to bind immune complexes. Bound immune complexes were collected by centrifugation and then washed three times by suspension in 1 ml of wash buffer and centrifugation (20 min at 8,500 rpm in an HB4 rotor) through 2 ml of wash buffer containing 30% sucrose. Bound immune complexes were transferred in 1 ml of wash buffer to a 1.5-ml tube and collected by centrifugation for 45 s in the microfuge. Immune complexes were recovered from the Formalin-fixed staphylococcus A cells as described for protein A-Sepharose-bound immune complexes, except that the elution buffer contained 5 to 10 μg of sonicated calf thymus DNA per ml.

Preparation of DNA. DNAs from immunoprecipitates and immunoprecipitation supernatants were precipitated by adding NaCl (final concentration, 0.2 M) and 2 volumes of ethanol and then incubating at −20°C overnight. The precipitated DNA was collected by centrifugation for either 15 min in an Eppendorf microfuge or 20 min at 9,000 rpm in an HB4 rotor. The precipitates were washed once with cold 70% ethanol and suspended in 200 to 400 μl of 10 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA–0.5% sodium dodecyl sulfate. RNase A was added to 100 μg/ml, and the samples were incubated for at least 1 h at 37°C. Proteinase K was added to 50 μg/ml, and the samples were further incubated at 37°C for at least 5 h. DNAs were ethanol precipitated, rinsed once with 70% ethanol, and then prepared for hybridization analyses.

DNAs that were dot blotted to nitrocellulose were dissolved in 397 μl of 56.7 mM Tris-hydrochloride (pH 7.4)–0.2 M NaOH–6.67× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and heated to 80°C for 10 min. The solution

was neutralized with 53 μl of 2 M Tris-hydrochloride (pH 7.4) and then applied to a nitrocellulose filter, previously wetted with 20× SSC, that was supported in a Hybridot manifold (Bethesda Research Laboratories). DNAs that were to be radiolabeled by nick translation were dissolved in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA–0.2 M NaCl, extracted with phenol-chloroform-isoamyl alcohol (50:50:1) and then with ether, ethanol precipitated, and finally dissolved in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA. DNAs that had been both restriction cut and precipitated with Formalin-fixed staphylococcus A cells were spermine precipitated (18) before electrophoresis in agarose gels containing Tris-borate buffer. The spermine precipitation was necessary to remove unidentified contaminating substances that caused the DNA to run anomalously on gels.

Hybridization analyses. DNAs to be blotted from agarose gels were treated as described previously (43), except that the DNA was depurinated with 0.06 M HCl for 30 min instead of with 0.25 M HCl for 30 min. DNA was blotted to nitrocellulose essentially as described previously (39). For one experiment (see Fig. 3), restriction fragments from cloned *D. melanogaster* DNA were blotted to diazobenzyl-oxymethyl-paper essentially as described previously (43). For two experiments, an amount of each clone calculated to give 150 to 200 ng of DNA in a 1-kilobase (kb) fragment was present on each filter (see Fig. 3 and 4). Radiolabeling of DNA was performed as described previously (33), and hybridization of radiolabeled DNA to filter-bound DNA was performed with dextran sulfate as described previously (43). Most hybridizations were performed overnight, but a few (see Fig. 3 and 4) were performed for 36 h to ensure that the majority of radiolabeled sequences annealed to their homologous cloned fragments on the filter. Autoradiography of all filters was performed with preflashed film in the presence of an intensifying screen.

RESULTS

Experimental design. Figure 1 outlines the experimental method that we have used to detect RNA polymerase II-DNA interactions in *D. melanogaster* cells. Cells were chilled on ice and then irradiated with UV light. By using a nitrocellulose filter-binding assay (41), we found that this treatment cross-linked an average of one protein molecule to DNA every 60 kb (data not shown). The DNA was then sheared to approximately 30 kb in length, and the nuclear DNA and covalently attached proteins were copurified on a CsCl gradient.

To identify the DNA sequences near the covalently coupled protein, the DNA was shortened either by sonication or by restriction endonuclease cleavage. The DNA attached to RNA polymerase II was purified by immunoprecipitation with anti-RNA polymerase II antibody. A portion of the DNA which failed to precipitate with the antibody was also purified and was used as a standard to estimate the fraction of a particular DNA sequence being immunoprecipitated. Since greater than 90% of the DNA remained in this supernatant, the sequence composition of this fraction was essentially that of total unfractionated DNA (data not shown); therefore the supernatant DNA is referred to as total DNA.

Immunoprecipitated and total DNAs were each treated with protease (and RNase) to remove covalently coupled proteins and then characterized by one of three assays. Sonicated DNA was characterized by either of two methods. In assay 1, the DNA was spotted directly on a nitrocellulose filter, and the filter was probed by hybridization with radiolabeled cloned DNA (dot blot). In assay 2, the DNA was radiolabeled by nick translation and hybridized to restriction fragments of cloned DNA that were bound in excess to nitrocellulose or diazobenzyloxymethyl-paper. In assay 3, restriction-cut DNA was characterized by Southern blot analysis (Fig. 1). In each case, comparison of the hybridization signals of immunoprecipitated and total DNAs provided a measure of the relative enrichment or depletion of particular sequences in the immunoprecipitate.

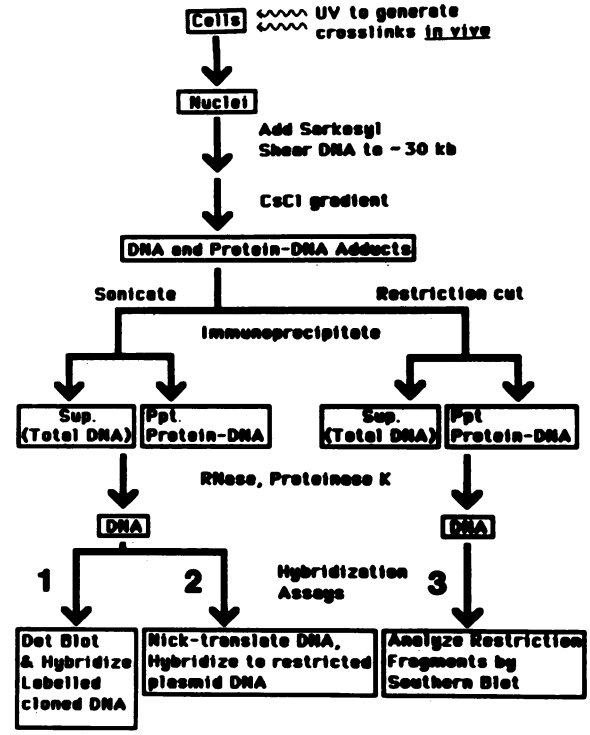

FIG. 1. Experimental design. Protein-DNA cross-links are generated in vivo with UV light and copurified with nuclear DNA, since the protein moiety contributes insignificantly to the mass of the protein-DNA adducts. DNA is shortened to an average size of 600 bp by random cleavage with sonication or to specific size fragments by cleavage with restriction endonucleases. Protein-DNA adducts are purified by immunoprecipitation of a specific protein and the covalently attached DNA is isolated and characterized by hybridization assays. DNA randomly broken by sonication is characterized by either assay 1 or assay 2. In assay 1, the DNA is spotted directly on nitrocellulose, and the presence of a specific sequence is detected with radiolabeled cloned DNA. In assay 2, the DNA itself is radiolabeled by nick translation and then hybridized to Southern blots of restriction digests of cloned *D. melanogaster* DNAs. In assay 3, immunoprecipitated DNA which was shortened with restriction enzymes is assayed by blotting the immunoprecipitated DNA from agarose gels to nitrocellulose (39) and detecting specific sequences on the nitrocellulose with radiolabeled cloned DNA. For all three assays, DNA is purified from a fraction of the immunoprecipitate supernatant (Total) and this DNA serves as a standard to quantitate the fraction of the DNA in the immunoprecipitates.

We anticipate that sequences transcribed at high levels by RNA polymerase II should be greatly enriched in the anti-RNA polymerase II immunoprecipitate. With assay 1, 30-fold more hsp70 gene sequence was detected in the anti-RNA polymerase II immunoprecipitate from heat-shocked cells than from non-heat-shocked cells (Fig. 2). In striking contrast, eightfold less of the copia sequence was in the immunoprecipitate from heat-shocked cells than in that from

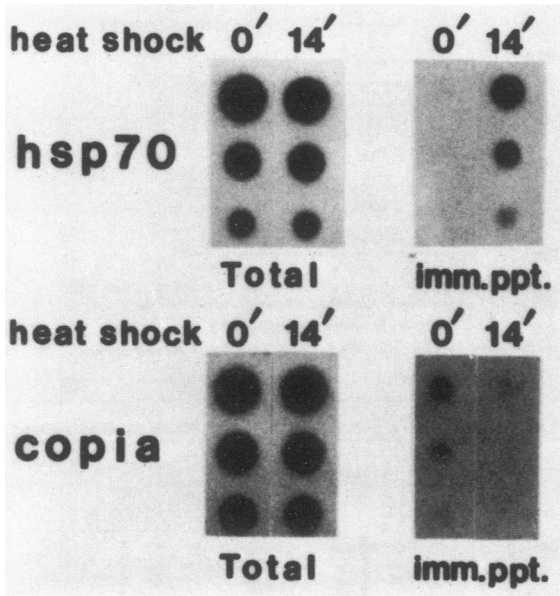

FIG. 2. Detection by dot blot hybridization of DNA sequences cross-linked to RNA polymerase II. *D. melanogaster* cells grown to 1.1×10^7 cells per ml were either not heat shocked (0′) or heat shocked by incubation for 14 min (14′) at 37°C and then UV irradiated for 10 min. DNA and covalently bound proteins were prepared from these cells and then sonicated and immunoprecipitated with antibody to RNA polymerase II. Total DNA (Total) from 10^6 cells and DNA immunoprecipitated with anti-RNA polymerase II antiserum (imm.ppt.) from 10^8 cells were bound to nitrocellulose with aliquots containing three- and ninefold less, respectively, of each DNA dotted in a descending series (dot blot). hsp70 gene sequences were detected by hybridization with the radiolabeled, 2.2-kb *Xba*I restriction fragment from 132E3 (27); this restriction fragment is homologous to 2 kb of the structural gene and 0.2 kb of the upstream sequences immediately flanking the start of transcription. Copia gene sequences were detected with the radiolabeled plasmid, cDm1142 (9); this plasmid contains one copy of the entire copia gene plus several kb of nonrepetitive DNA.

non-heat-shocked cells. This agrees quite well with transcription measurements, which show that hsp70 transcription is activated 150-fold (24) and copia transcription is repressed sixfold (28) after heat shock.

Several control experiments (data not shown) demonstrated that this method detects only DNA sequences which are associated with RNA polymerase II. (i) The immunoprecipitation of DNA with anti-RNA polymerase II antiserum required UV treatment of cells; thus, protein must be cross-linked to DNA to detect the RNA polymerase II-DNA interactions. (ii) The sensitivity of the hybridization signals to DNase I but not to RNase A indicates that the protein is not coupled to the DNA via an RNA bridge. (iii) The hsp70 and actin (see Fig. 6) gene sequences were also precipitated with an antiserum against the isolated 215,000-dalton subunit of RNA polymerase II but not precipitated with an antiserum against *Escherichia coli* RNA polymerase, indicating that precipitation of the DNA is due to its association with RNA polymerase II. (iv) Sequences homologous to ribosomal RNA, which is transcribed by RNA polymerase I, were depleted in the anti-RNA polymerase II precipitates (see Fig. 3), indicating that the mere act of transcription is not sufficient for the precipitation of sequences with anti-RNA polymerase II antiserum.

The distribution of RNA polymerase II on specific genes in heat-shocked and non-heat-shocked cells. The association of RNA polymerase II with many genes can be measured simultaneously by radiolabeling the immunoprecipitated DNA and then hybridizing this DNA to Southern blots of restriction fragments of cloned *D. melanogaster* DNA (Fig. 1, assay 2). Since the restriction fragments on the filter are in at least 100-fold excess over the radiolabeled sequences, the amount of radiolabeled DNA hybridizing to each restriction fragment is proportional to the relative amount of its homologous sequences in the radiolabeled, immunoprecipitated DNA.

Figure 3 shows large differences in the relative amounts of particular DNA sequences in preparations of DNA from the immunoprecipitate supernatant (Total) and the anti-RNA polymerase II immunoprecipitates (NHS imm. ppt. and HS imm. ppt.). The hybridization signals of the total DNA reflect the genomic repetition frequency of each DNA: 250 for ribosomal gene (rDNA) (14, 47), 100 for each histone gene (22), ca. 100 for copia (30), 5 for hsp70 (48), 6 for actin (11), and 1 each for hsp83 (28), hsp26, hsp27, and hsp23 (hsp20s) (19, 38). Also, as indicated by the hybridization with total DNA, a moderately repeated sequence must reside on the 2.1-kb restriction fragment in the hsp70 lane.

DNA sequences encoding mRNAs transcribed by RNA polymerase II are enriched in the anti-RNA polymerase II immunoprecipitates. The heat shock gene sequences of hsp83, hsp70, hsp26, hsp27, and hsp23 are all enriched in anti-RNA polymerase II immunoprecipitates from heat-shocked cells at least several hundredfold relative to the rDNA sequences. The hsp83 gene is also enriched in the immunoprecipitate from non-heat-shocked cells, as expected, since this gene is transcribed at moderate levels in non-heat-shocked cells (24). In addition, the histone, copia, and actin gene sequences are enriched in immunoprecipitates from non-heat-shocked cells; each of these genes as well as the heat shock genes will be discussed in more detail below.

Boundaries of RNA polymerase II association. Hsp26, hsp83, and their flanking sequences are well characterized and present in a single copy per genome (28, 38). Thus, the analyses of RNA polymerase II distribution by assay 2 (Fig. 1) provide an opportunity to determine whether RNA polymerase II initiates binding near the promoter of these genes in heat-shocked cells, since the assay is not complicated by the presence of repeated DNA. For hsp83, the hybridization signal from the 525-base-pair (bp) *Xba*I fragment from the 5′ transcribed region was 2.3-fold more intense than the hybridization signal from the 800-bp *Bam*HI-*Xba*I fragment immediately upstream of the start of transcription (Fig. 4, lane a). Hybridization to the 2.3-kb *Bam*HI-*Eco*RI fragment containing the hsp26 gene was at least 10 times more intense than was hybridization to the 2.6-kb *Eco*RI fragment immediately upstream of its start of transcription (Fig. 4, lane b). (The 2.6-kb fragment contains sequences homologous to a developmentally regulated gene, gene 1, but this gene is not expressed in Schneider line 2 cells [38].) Thus, the region of high RNA polymerase II density begins within a few hundred base pairs (bp) of the hsp26 and hsp83 promoters.

Transcription termination for at least one of the hsp70 genes can also be assessed. In Fig. 3B (HS imm. ppt., hsp70), the hybridization of radiolabeled, immunoprecipitated DNA from heat-shocked cells to a 470-base-pair *Hin*cII fragment immediately downstream of the polyadenylate [poly(A)] addition site was 50-fold less intense than the hybridization to a 900-base-pair *Bam*HI-*Hin*cII fragment

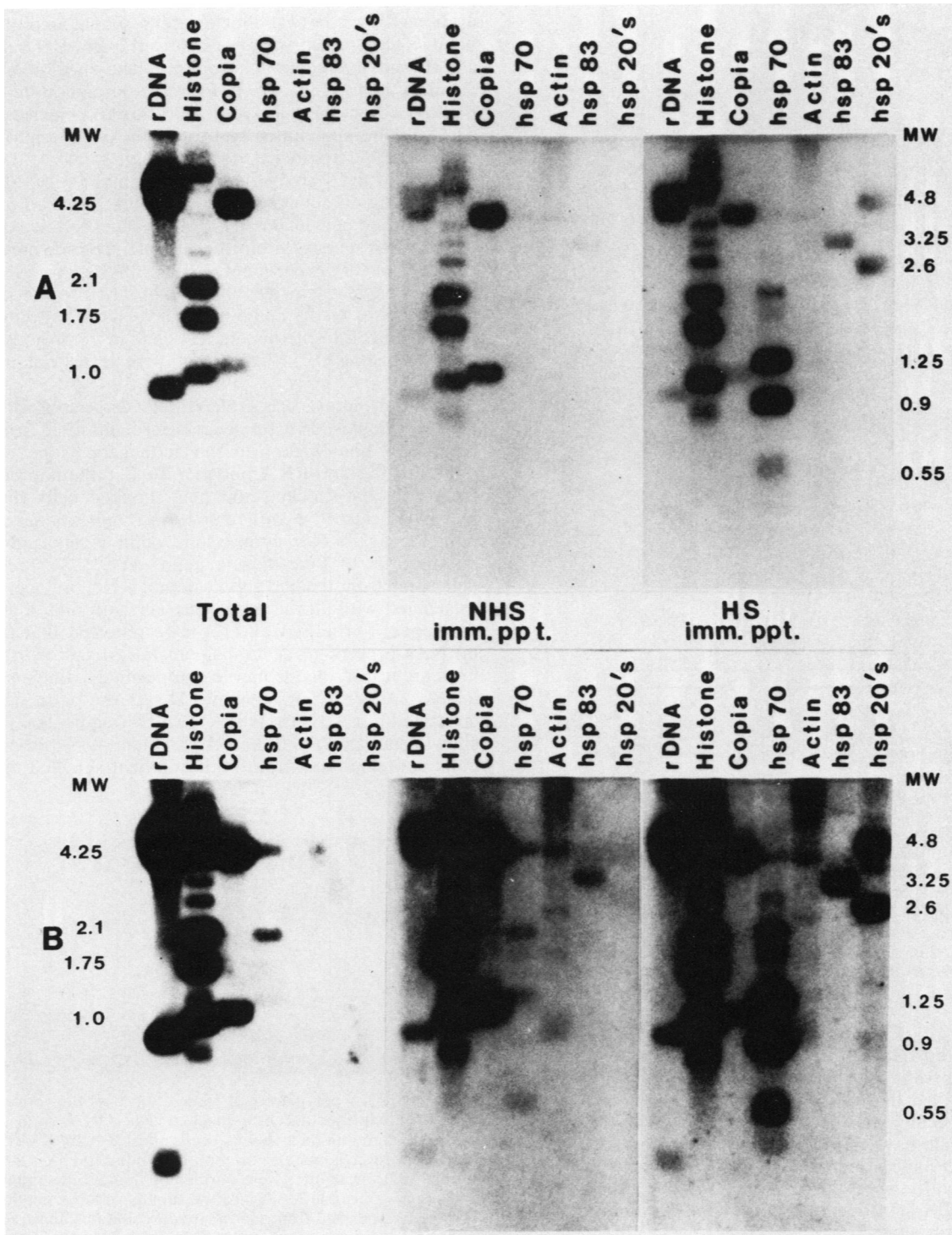

FIG. 3. Hybridization of radiolabeled total and immunoprecipitated DNA to restriction fragments from cloned *D. melanogaster* DNA. Total DNA (50 ng) from UV-irradiated, non-heat-shocked cells (Total) and DNA immunoprecipitated with anti-RNA polymerase II antiserum from 10^8 heat-shocked cells (HS imm.ppt.) or from 10^8 non-heat-shocked (NHS imm.ppt.) cells were radiolabeled by nick translation. Total DNA (5×10^6 cpm) and 1.2×10^6 cpm of each of the immunoprecipitated DNAs were separately hybridized to identical filters containing restriction-cut clones of *D. melanogaster* DNA. (A) The autoradiographic exposures of the filters were as follows: total DNA, exposed for 4 h; both immunoprecipitated DNAs, exposed for 24 h. (B) Autoradiographs resulting from longer exposures of the same filters as in panel A were as follows: total DNA, exposed for 12 h; both immunoprecipitated DNAs, exposed for 5 days. Sequences homologous to the genes identified above each lane are present on restriction fragments of the following sizes (the positions of some molecular weights are given on the two sides of panels A and B): rDNA (*Hin*dIII-*Eco*RI-cut DmrY22), 6, 4.35, 0.87 and 0.3 kb; histone (*Hin*dIII-*Ava*I-cut cDm500), 2.1, 1.75 and 1 kb; copia (*Hha*I-cut cDm1142), 4.25 and 1.2 kb; hsp70 (*Hin*cII-*Bam*HI-cut 56H8), 1.25, 0.9, 0.55 and 0.47 kb; actin (*Sal*I-cut DmA2), 1.1 and 0.9 kb; hsp83 (*Bam*HI-*Sal*I-cut aDm4.46), 3.25 kb; hsp20s (*Eco*RI-cut 202.7), 4.8 (contains hsp23 and hsp27), 2.6 (two comigrating fragments containing hsp26 and gene 1), 1 and 0.8 kb. Low levels of DNA homologous to pBR322 and *E. coli* DNA contaminated the preparations of immunoprecipitated DNA, accounting for hybridization to the 4.1-kb restriction fragments in lanes hsp83 and hsp20's and to DNA at the top of the actin lane.

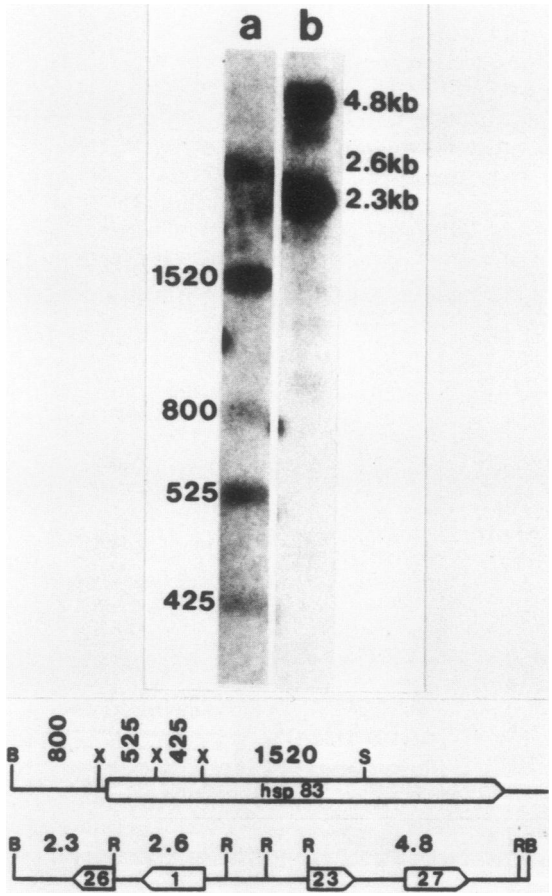

FIG. 4. Further delimitation of the distribution of RNA polymerase II on hsp26 and hsp83. Radiolabeled immunoprecipitated DNA from heat-shocked cells was hybridized to restriction fragments homologous to hsp83 (lane a, *Bam*HI-*Xba*I-*Sal*I-cut aDm4.46) and to the small heat shock genes (lane b, *Bam*HI *Eco*RI-cut 202.7). The fragments homologous to hsp83 (first restriction map) are 1,520, 800, 525, and 425 bp in size. The three fragments homologous to the region containing the small heat shock genes (second restriction map) are 4.8, 2.6, and 2.3 kb in size. The gene labeled 1 is a developmentally regulated gene that is not expressed in these cells. Abbreviations: B, *Bam*HI; X, *Xba*I; S, *Sal*I; R, *Eco*RI.

immediately upstream of the poly(A) addition site. Since the 470-base-pair fragment is homologous to only one of the five hsp70 genes and the 900-bp fragment is homologous to all five, one can calculate that the hybridization to the 470-bp fragment would have been only 10-fold less than the hybridization to the 900-bp fragment had RNA polymerase II been associated with the entire 470-bp fragment. Thus, RNA polymerase II is not detected far beyond the poly(A) addition site of this gene.

The average density of RNA polymerase II on the histone repeat is approximately the same in non-heat-shocked and heat-shocked cells. The histone sequences are clearly enriched in the RNA polymerase II immunoprecipitates from both heat-shocked and non-heat-shocked cells (by comparison to the 0.87-kb rDNA fragment in Fig. 3A). Comparable levels of histone transcripts (averaged over the five genes) were detected in heat-shocked and non-heat-shocked cells (28). Thus, the histone sequences could provide an internal standard for comparing the radiolabeled, immunoprecipitated DNA preparations from heat-shocked cells with preparations from non-heat-shocked cells. Equal amounts of the histone gene repeat were present in the anti-RNA polymerase II immunoprecipitates from non-heat-shocked and heat-shocked cells (Fig. 5). Before the immunoprecipitation, nuclear DNAs were cleaved with the endonuclease *Xho*I. The immunoprecipitated and total DNAs were purified and examined by Southern blotting (Fig. 1, assay 3). Equal amounts of the 5-kb *Xho*I fragment containing all five genes were immunoprecipitated from the heat-shocked and non-heat-shocked cells. Compensating changes in the expression of individual genes within the histone repeat would not be detected in this experiment. When different restriction enzymes were used to cleave the DNA before the immunoprecipitation, we did find reduced levels of RNA polymerase II cross-linked to the histone H1 gene in heat-shocked cells (data not shown), in agreement with transcript measurements (7).

RNA polymerase II is preferentially associated with one of the two cytoplasmic actin genes before and after heat shock. Sequences homologous to the actin gene clone were enriched in the anti-RNA polymerase II immunoprecipitates from non-heat-shocked and heat-shocked cells (Fig. 3B). However, since the *Sal*I restriction fragments are strongly homologous to two cytoplasmic actin genes and weakly homologous to four muscle actin genes (11), we cannot determine from these results whether RNA polymerase II is associated with all the actin genes or with only a subset of the genes. Fyrberg et al. (12) have reported that the actin mRNAs present in several *D. melanogaster* cultured cell lines are transcribed from the cytoplasmic actin genes at the locus 42A or 5C. In genomic DNA, the actin genes are contained on six resolvable *Eco*RI fragments. Figure 6 shows that an 8.7-kb *Eco*RI fragment was preferentially immunoprecipitated from both non-heat-shocked and heat-

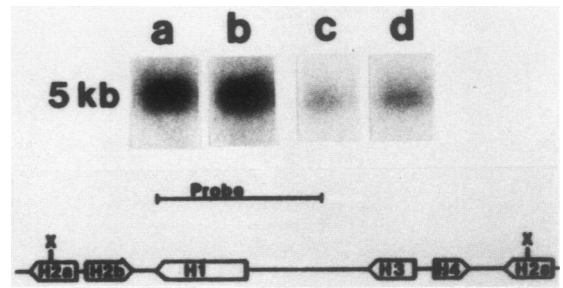

FIG. 5. RNA polymerase II association with the histone repeat in non-heat-shocked and heat-shocked cells. DNAs from 1.8×10^8 UV-irradiated, non-heat-shocked cells and 1.8×10^8 UV-irradiated, heat-shocked cells were restriction cut with *Xho*I (X). RNA polymerase II-DNA adducts were purified by immunoprecipitation with antiserum against the 215,000 dalton subunit of RNA polymerase II. DNAs were purified from the immunoprecipitates and fractionated on a 1% agarose gel along with 5.5% of the DNA purified from the immunoprecipitate supernatants. All DNAs were blotted to nitrocellulose, and histone sequences were detected by hybridizing the blots with the radiolabeled 1.9-kb *Hpa*I-*Bgl*II restriction fragment purified from plasmid cDm500. Lanes contained immunoprecipitate supernatants from non-heat-shocked (lane a) and heat-shocked (lane b) cells and immunoprecipitates from non-heat-shocked (lane c) and heat-shocked (lane d) cells. The locations of the *Xho*I sites whose cleavage results in the 5-kb restriction fragment are shown on the restriction map below the autoradiograph. The autoradiographic exposure of the immunoprecipitate supernatant samples (lanes a and b) was 1 h and the exposure of the immunoprecipitated samples (lanes c and d) was 12 h.

shocked cells with anti-RNA polymerase II antiserum (assay 3). This fragment contains the cytoplasmic actin derived from the 5C locus. The 6-kb fragment containing the cytoplasmic actin gene located at 42A and the 1.2- and 14-kb fragments containing muscle actin genes were not detected in this immunoprecipitate or in precipitates from a repeat of this experiment in which the 8.7-kb fragment was overexposed (data not shown). The remaining two muscle actins were not detected at the stringency of this hybridization.

DISCUSSION

The distribution of a protein on DNA determined in vivo: sensitivity and resolution. The protein-DNA cross-linking method described here has sufficient sensitivity to detect RNA polymerase II on single-copy heat-shock genes. Assuming that RNA polymerase II (ca. 500,000 daltons [51]) covers approximately 50 bp of DNA (calculated assuming a protein of globular shape), then no more than 20 molecules could occupy a 1-kb heat shock gene. Since the presence of RNA polymerase II on a single heat shock gene is easily detected by using 10^7 cells (Fig. 3), use of 20 times more cells (2×10^8 cells) should permit detection of a single protein associated with single-copy DNA if the protein cross-links to DNA with an efficiency comparable to that of RNA polymerase II.

The amount of nonspecifically bound DNA recovered in the immunoprecipitates is very low, since the hsp70 sequences are enriched almost 1,000-fold relative to rDNA sequences in the immunoprecipitates from heat-shocked cells. The low level of rDNA sequences detected may be a measure of the level of nonspecific precipitation inherent in this procedure. Alternatively, the observed background may be caused by a low level of RNA polymerase II binding to nucleosomes of transcriptionally active rDNA genes (2), by the antiserum cross-reacting weakly with RNA polymerase I, or by weak RNA polymerase II promoters residing within the rDNA gene repeat.

We have described three assays that may be used to detect DNA sequences in immunoprecipitates of RNA polymerase II-DNA adducts. In assay 1, the DNA is applied directly onto nitrocellulose (dot blot) and the presence of specific sequences is detected by hybridization with specific radiolabeled DNA. This assay is rapid, but one is limited to examining one sequence per filter per hybridization.

In assay 2, the protein distribution on many sequences can be determined simultaneously by hybridizing radiolabeled, immunoprecipitated DNA to DNA (Southern) blots of restriction enzyme-cut plasmids. This is convenient for obtaining an overview of the distribution of a protein on several genes but has limited resolution. As one attempts to maximize resolution by reducing the size of the immunoprecipitated DNA, sensitivity decreases because the short DNA is not nick translated as efficiently and because less DNA is coupled to each RNA polymerase II molecule. Also, when the DNA sequence on the clone is present in multiple copies in the genome, it is difficult to distinguish whether the protein is associated with each copy, with a few of the copies, or with sequences that flank the copies.

In assay 3, the protein distribution is measured on fragments generated by restriction endonuclease digestion. The resolution of this method is limited only by the position of restriction sites and by the inactivity of some restriction enzymes in the detergents required for DNA isolation. Sensitivity is high, since the hybridization probe can be as large as the restriction fragment to be detected, and each copy of a repeated sequence can be examined by capitalizing either on restriction site polymorphisms within the repeated sequence or on restriction site differences in the flanking regions of each copy of the repeated sequence.

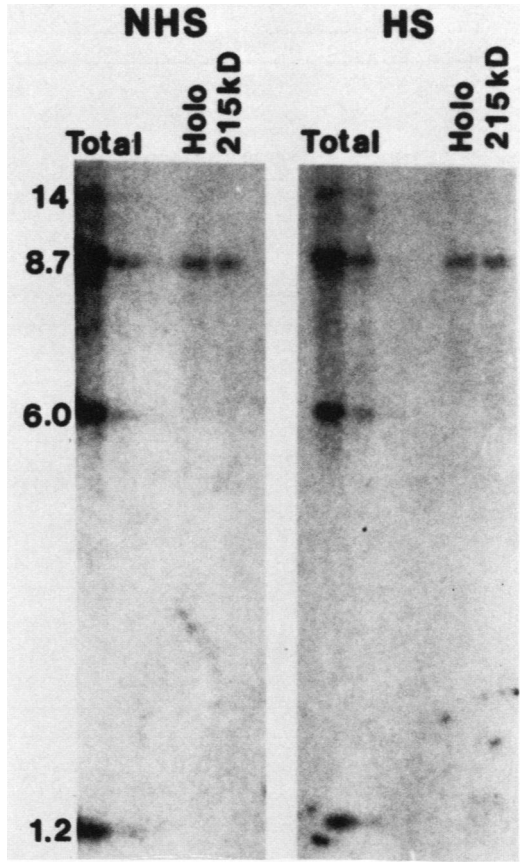

FIG. 6. RNA polymerase II association with the actin genes in non-heat-shocked and heat-shocked cells. DNAs from 6×10^7 UV-irradiated, non-heat-shocked cells (NHS) and 6×10^7 UV-irradiated, heat-shocked cells (HS) were cleaved with *Eco*RI. RNA polymerase II-DNA adducts were purified by immunoprecipitation with antiserum against either purified RNA polymerase II (Holo) or the 215,000-dalton subunit of RNA polymerase II (215 kD). DNA from each immunoprecipitate was fractionated on a 1% agarose gel along with 3.3, 1.1, and 0.36% of the DNA from the immunoprecipitation supernatant (total) of each sample. All the DNAs were blotted to a nitrocellulose filter. Actin sequences were detected with a radiolabeled 0.9-kb *Sal*I restriction fragment from the plasmid DmA2.

Correlation between the presence of RNA polymerase II and transcript abundance. Figure 7 provides a quantitative summary of the distribution of RNA polymerase II on several genes in non-heat-shocked and heat-shocked *D. melanogaster* cells as derived from the data in Fig. 3 and refined by other described experiments. Above each fragment is the density of RNA polymerase II associated with the fragments in non-heat-shocked cells; below each fragment is the corresponding density in heat-shocked cells. The density refers to the number of RNA polymerase II molecules per kb of DNA.

The density of RNA polymerase II on several genes correlates very well with transcription rates measured by pulse-labeling experiments. By using the cross-linking assay, we observe that the density of RNA polymerase II increases 30-fold on the small heat shock genes and 100-fold on the 3′ half of hsp70 in heat-shocked cells. The amount of RNA

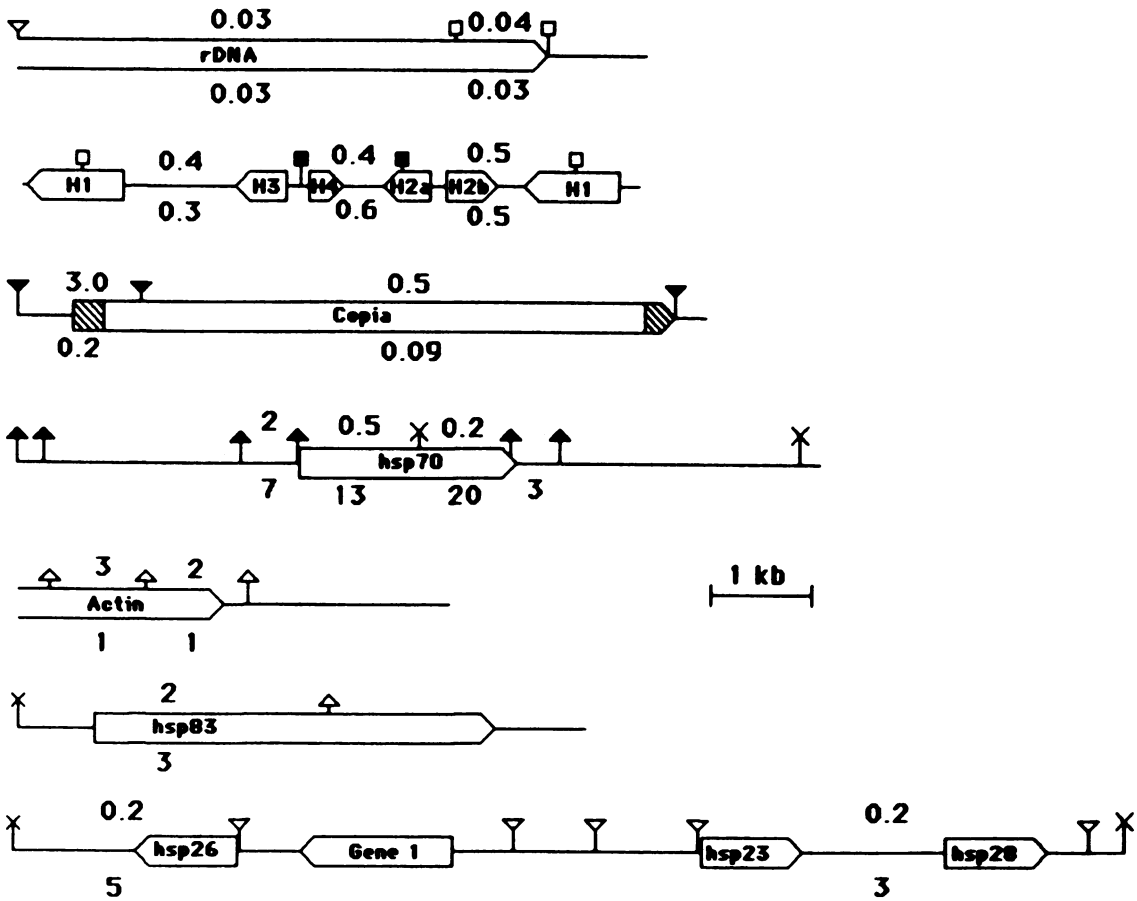

FIG. 7. Calculated densities of RNA polymerase II on specific genes in non-heat-shocked and heat-shocked cells. The amounts of specific DNA sequences in the anti-RNA polymerase II immunoprecipitates from heat-shocked and non-heat-shocked cells were quantitated by densitometric measurements of the blots in Fig. 3. These values were corrected for the copy number and size of each restriction fragment so that the number printed above (non-heat shock) and below (heat shock) represents the absolute number of RNA polymerase II per kb present on each section of DNA. Since only a fraction of the RNA polymerase II molecules cross-linked to the DNA, the absolute density of RNA polymerase II molecules was estimated relative to the hsp70 gene in heat-shocked cells. The high level of transcripts produced from hsp70 requires that RNA polymerase II be packed at approximately 10 to 20 molecules per kb (21). We used the value of 20 RNA polymerase II molecules per kb, which is probably the upper limit, for our estimations of RNA polymerase II density. The values obtained from the different hybridizations were corrected by using the histone genes as an internal standard (see the text and Fig. 5). RNA polymerase II is assumed to be associated with only one actin gene (see the text and Fig. 6) and associated equally with the members of the other multigene families. Arrows depict known transcripts. Restriction maps for each gene are derived from the sources described in the text. Symbols: ×, *Bam*HI; ▽, *Eco*RI; ▼, *Hha*I; □, *Hin*dIII; ■, *Ava*I; △, *Sal*I; ▲, *Hin*cII.

transcribed from the genes for hsp70, hsp26, hsp27, and hsp23 increases approximately 150-fold after heat shock (24) (literature values vary from 20-fold [8] to 1,000-fold [42].)

The amount of RNA polymerase II associated with the entire histone repeat changes very little in response to heat shock (Fig. 5). This agrees both with measurements of histone RNA synthesis (28) and with similar labeling of the histone loci upon hybridization of pulse-labeled RNA from heat-shocked and non-heat-shocked cells to polytene chromosomes (40).

Expression of most genes is repressed by heat shock. The 5.5-fold decrease in the density of RNA polymerase II on the copia gene after heat shock (Fig. 3 and 7) indicates that the repression in this case is at the level of RNA polymerase II interaction with the copia gene. This agrees well with the sixfold decrease in the synthesis of copia RNA reported after heat shock (28).

Not all of our measurements of RNA polymerase II density agree quantitatively with previous measurements of RNA synthesis rates. Findly and Pederson (8) have reported that actin transcription detected by 5-min pulse-labeling of RNA with radioactive precursors decreases 15-fold after heat shock of *D. melanogaster* cells. In contrast, we observed an average decrease (Fig. 3 and 6) of only 1.5-fold after heat shock in the amount of RNA polymerase II associated with actin. This apparent discrepancy may reflect technical differences in the two types of experiments. The pulse-labeling measurements required pretreatment of the cells with 5-fluorouridine to incorporate sufficient levels of radionucleotides into the RNA for its detection. Moreover, the heat shock involved a very rapid transition to 37°C, whereas our heat shock was more gradual, occurring over a period of a few minutes. The latter difference is known to influence the heat shock response (23). Alternatively, we cannot rule out that the stability of the actin primary transcript might decrease during heat shock.

We detect low levels of RNA polymerase II on hsp70 and the small heat shock genes in non-heat-shocked cells. For hsp70, the RNA polymerase II is concentrated on the 5′ half of the gene. In Fig. 3B (NHS imm. ppt., hsp70), 3.5-fold more radiolabeled, immunoprecipitated DNA from non-heat-shocked cells hybridizes to the 1.25-kb restriction fragment homologous to the 5′ half of hsp70 than to the 0.9-kb restriction fragment homologous to the 3′ half of the gene. Moreover, hybridization to the 0.55- and 1.25-kb restriction fragments is nearly equal even though the 0.55-kb restriction fragment is homologous to only 0.2 kb immediately upstream of the transcription start of all five hsp70 genes and the 1.25-kb fragment is homologous to virtually all 1.25 kb immediately downstream of the transcription start of all five hsp70 genes (20). Figure 3A (NHS imm. ppt., Copia) shows that more RNA polymerase II may also be present on the 5′ end of the copia gene than on the remainder of the gene because the level of hybridization to the 1.2-kb fragment containing the copia promoter (10) is nearly equivalent to the level of hybridization to the 4.25-kb *Hha*I fragment containing most of the copia structural gene. We are now evaluating the possibility that RNA polymerase II is concentrated on these promoters.

Defining the sites of transcription initiation and termination in vivo. The distribution of RNA polymerase II on hsp83 and hsp26 indicates that RNA polymerase II initiates contact with DNA near the transcription initiation site. DNase I footprinting in vitro by Parker and Topol (29) and exonuclease III protection studies in intact nuclei by Wu (49) indicate that a protein-DNA complex protects DNA from −40 to +30 of at least some of the inactive heat shock genes. This region is highly conserved among heat shock genes (16). Thus, our observations are consistent with the hypothesis that RNA polymerase II initiates contact near the binding sites of these potential transcription factors.

We have also examined the extent of the RNA polymerase II association at the 3′ end of the hsp70 gene. Hofer and Darnell (17) have shown that the site of transcription termination of the mouse globin gene is as much as 1 kb downstream of the poly(A) addition site. In contrast, we estimate that termination occurs within 200 bp of the poly(A) addition site of the hsp70 gene because only a low level of immunoprecipitated DNA from heat-shocked cells hybridizes to a restriction fragment from this region (Fig. 3B). This result agrees with the nuclear runoff experiments of Miller and Elgin (26). The contrast with the mouse globin gene result suggests that the distance between the sites of transcription termination and poly(A) addition may be quite variable for different genes.

Prospectus. The method described here provides a new way to examine chromatin structure in the cell. Its two requirements are that antibody be available to the protein of interest and that the protein cross-link to the DNA at detectable levels. Having met these requirements, application of the method provides a measure of protein-DNA interactions in their natural configurations. This should be of considerable help in elucidating the role of specific protein-DNA interactions in chromosome function.

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